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Plasma von Willebrand Factor Antigen (vWF:AG) and Thrombomodulin (TM) Levels in Adult Thrombotic Thrombocytopenic Purpura/Hemolytic Uremic Syndromes (TTP/HUS) and Bone Marrow Transplant-Associated Thrombotic Microangiopathy (BMT-TM)

Z.R. Zeigler, C.S. Rosenfeld, D.F. Andrews III, J. Nemunaitis, J.M. Raymond, R.K. Shadduck, R.E. Kramer, J.F. Gryn, P.B. Rintels, E.C. Besa, and J.N. George

Special Hematology Laboratory, Western Pennsylvania Cancer Institute, Western Pennsylvania Hospital, Pittsburgh, Pennsylvania (Z.R.Z., C.S.R., D.F.A., J.N., J.M.R., R.K.S.,); Contributing members of the North American TTP Group, Pittsburgh, Pennsylvania (Z.R.Z., C.S.R., D.F.A., J.N., J.M.R., R.K.S., R.E.K., J.F.G., P.B.R., E.C.B., J.N.G.,); Johnstown Regional American Red Cross, Johnstown, Pennsylvania (R.E.K.); Cooper Hospital, Camden, New Jersey, (J.F.G.); Rhode Island Hospital, Providence, Rhode Island (P.B.R.); Medical College of Pennsylvania, Philadelphia, Pennsylvania (E.C.B.); University of Oklahoma Health Science Center, Oklahoma City, Oklahoma (J.N.G.)

Endothelial damage is thought to be a contributing factor in the pathogenesis of Thrombotic Thrombocytopenic Purpura/Hemolytic Uremic Syndromes (TTP/HUS). The present studies measured two markers of endothelial cell stimulation and/or activation [von Willebrand Factor (vWF:Ag) and thrombomodulin (TM)] in patients with TTP/HUS disorders and compared them to controls. The patient groups consisted of adults with TTP/HUS, with (n = 13) and without (n = 14) peak Cr levels >2.0 mg/dl. Additionally, 52 patients with Bone Marrow Transplant-associated Thrombotic Microangiopathy (BMT-TM) following allogeneic BMT were evaluated. Both vWF:Ag and TM were elevated in all patient groups compared to controls. TTP/HUS patients with peak Cr >2.0 mg/dl had higher TM levels ($P < 0.001$) than did those with peak Cr levels below 2 mg/dl. However, thrombomodulin/creatinine (TM/Cr) ratios did not differ in these two groups nor did they differ from controls. BMT-TM pts had higher vWF:Ag levels and higher TM/Cr ratios than controls and TTP/HUS, $P < 0.001$. The median TM/Cr ratio in BMT-TM was 91 (range = 34–229) compared to 38 (range = 29–50) in controls, $P < 0.001$ and 38 (range = 6 to 156) in TTP/HUS, $P < 0.001$. Additionally both TM ($P < 0.001$) and TM/Cr ($P < 0.02$) were higher in patients with Grades 3 and 4 BMT-TM compared to those with Grade 2 BMT-TM. These results suggest that endothelial cell activation occurs in TTP/HUS and BMT-TM. Since TM/Cr ratios were higher in BMT-TM compared to TTP/HUS, these findings suggest that the mechanism of elevated TM in BMT-TM cannot be explained solely by altered renal excretion. Taken together, these findings strongly indicate a role of endothelial cell damage in BMT-TM.

Key words: TTP/HUS, von Willebrand factor, thrombomodulin

INTRODUCTION

Thrombotic Thrombocytopenic Purpura/Hemolytic Uremic Syndromes (TTP/HUS) are thrombotic microangiopathic disorders in which platelet and von Willebrand factor (vWF) rich thrombi are found within the microcirculation [1–3]. Historically these syndromes were defined by the type of organ dysfunction superimposed upon the basic diagnostic dyad of microangiopathic hemolytic anemia (MAHA) and thrombocytopenia. TTP was initially defined as the pentad of MAHA, thrombocytopenia, neurologic defects, renal dysfunction, and fever [4]. By contrast, HUS was initially defined as prominent renal dys-

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Address reprint requests to Zella R. Zeigler, M.D., Western Pennsylvania Hospital, Western Pennsylvania Cancer Institute, 4800 Friendship Avenue, Pittsburgh, PA 15224.

function, with little in the way of neurologic findings, superimposed upon MAHA and thrombocytopenia [5]. However, it has been observed that many TTP patients lack one or more features of the pentad and that patients with atypical HUS may have neurologic symptoms and fever [6–9]. Therefore, it has been suggested that these syndromes may represent a spectrum of disorders rather than separate entities [10,11]. A secondary thrombotic microangiopathy occurs in association with bone marrow transplantation and the use of cyclosporin A [12–19]. The etiology of bone marrow transplant associated thrombotic microangiopathy (BMT-TM) is unknown.

Possible etiologic factors for primary TTP/HUS disorders have included the presence of platelet agglutinins [20–23] that interact with von Willebrand factor (native or altered), or its multimers [24,25]. Others propose a role for endothelial damage caused by complement fixing antibodies [26,27] or Verocytotoxins [10,28,29] (the pathogenic agent in epidemic childhood HUS), which may also account for some cases of adult TTP/HUS.

von Willebrand Factor antigen (vWF:Ag) [30,31] and thrombomodulin (TM) [32–34] are considered to be markers of endothelial cell damage. However vWF:Ag is released from endothelial cells by physiologic [35,36] as well as by damaging stimuli. In contrast, TM appears to be released by damaging insults only [37–39]. Plasma TM levels have been reported as increased in TTP, in disseminated intravascular coagulation (DIC) [33,34], and with certain renal diseases [40]. Therefore the present studies measured vWF:Ag and TM at diagnosis in a series of adult patients with TTP/HUS, and in patients with BMT-TM.

METHODS

Excess citrated platelet poor plasma (that had been collected at diagnosis for routine coagulation assays) was used for these measurements. The plasma had been frozen at −70°C prior to analysis. Twelve/twenty-seven patients were participants in the North American TTP Study Group (NATG). This is a multi-institutional group that is conducting a trial to compare the effect of apheresis using either whole plasma (fresh frozen plasma) or the cryosupernatant fraction of plasma (cryosup) for replacement. All NATG patients gave informed consent for participation in this clinical study, which was approved by the individual participating institutional review boards. The remaining patients were consecutive TTP/HUS patients that were cared for by the physicians of the Western Pennsylvania Cancer Institute. The TTP/HUS disorder was unrelated to pregnancy, malignancy, HIV disease, or transplanation. These patients were treated in a similar fashion except the majority were exchanged with whole plasma. Their plasma samples consisted of residual plasma frozen at the time of routine coagulation studies at diagnosis. The BMT-TM patients were participants in a prospective study of BMT-TM. This protocol was approved by the Institutional Review Board of the Western Pennsylvania Hospital.

Twenty laboratory controls (ten males and ten females) had citrated platelet poor plasma collected and frozen at −70°C for the same assays. The females were not pregnant and were not taking oral contraceptives.

von Willebrand Factor Antigen (vWf:Ag)

vWF:Ag was quantified by an enzyme-linked immunosorbent assay using a sandwich technique (Diagnostica Stago, Asnieres-sur-Seine, France) [41]. The intra-assay and inter-assay coefficients of variation for the vWF:Ag assay were 2.8 and 4.7%, respectively.

Plasma Thrombomodulin (TM) Level

Plasma TM levels were also quantified by an enzyme-linked immunosorbent assay using a sandwich technique. The reagents for the thrombomodulin assay were kindly supplied by Diagnostica Stago [42]. The intra-assay and inter-assay coefficients of variation were 7.6 and 11.5%, respectively. Thrombomodulin is also excreted in the urine; therefore, a ratio of thrombomodulin/creatinine was calculated, to provide a measure of renal contribution to the TM elevation, as previously described by Tomura et al. [40].

STATISTICS

The Kruskal Wallis Anova by ranks test was used to compare the independent patient and control groups. Chi Square analysis in a Fisher's Exact Test was used to compare demographic features of the independent TTP/HUS groups. Correlation coefficients were calculated using the Pearson product moment correlation by casewise deletion of missing data. A P value < 0.05 was considered to be significant.

Box and Whisker plots are used in Figures 1–3. In these plots, the median is indicated by a square point in the center of the box. The box represents the 25 and 75% quartiles and the error bars denote the range of values in the independent patient and control groups.

METHODS

Determination of % Fragmented Erythrocytes

A single observer counted 500 red blood cells on blinded smears. The % fragmented red cells was then calculated as previously described [1].

Grading System for BMT-TM

BMT-TM was graded according to a grading system based upon lactic dehydrogenase level (LD) and % fragmented erythrocytes as previously described [12]. This

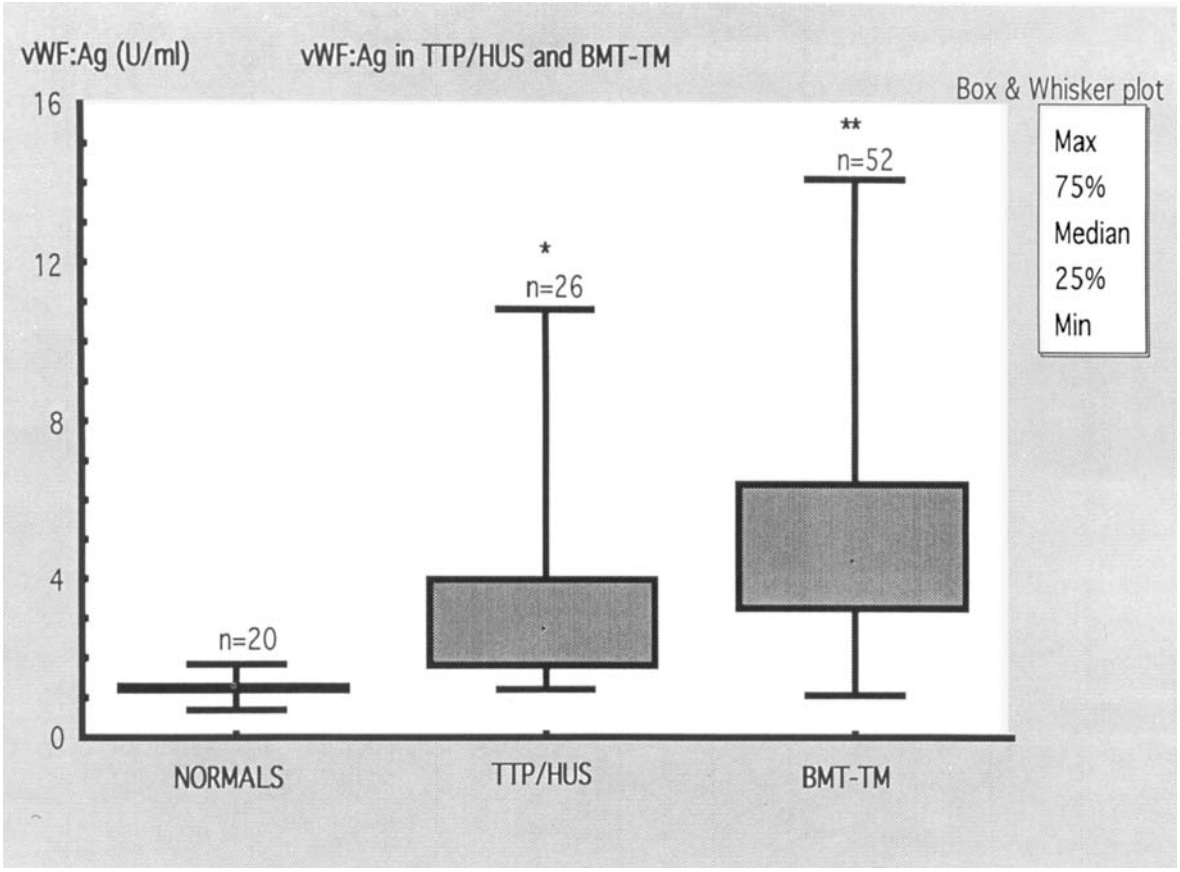

Fig. 1. Box and Whisker plot of vWF:Ag level at diagnosis in TTP/HUS, BMT-TM, and Normal control subjects. n = number. *$P < 0.001$ vs. Normals; **$P < 0.001$ vs. Normals and TTP/HUS.

system was designed to reflect activity of BMT-TM ranging from no evidence, to subclinical, to clinical BMT-TM of varying severity (see Table I).

GVHD Prophylaxis

Cyclosporine A (CsA) and methylprednisolone were used as GVHD prophylaxis in the allo-BMT patients. In unrelated donor transplants, methotrexate was also given in doses of 15 mg/M^2 on day +1 and of 10 mg/M^2 on days +3 and +6 as additional GVHD prophylaxis. Patients received CsA at 2.5 mg/kg every 12 hr IV from day-1 to discharge. Post-discharge, CsA was administered at 7.5 mg/kg po bid and tapered to 0.5 mg po bid at day 354 and discontinued at day 360 post-BMT. CsA doses were adjusted to maintain whole blood levels between 350 and 600 mcg/l, as measured by the polyclonal fluorescence polarization assay [43].

Corticosteroids were administered according to the following schedule: Methylprednisolone 0.25 mg/kg IV every 12 hr from 7–14 post-BMT, 0.5 mg/kg IV every 12 hr day 15–28, then oral Prednisone 0.4 mg/kg twice daily day 29–42, 0.25 mg/kg twice daily day 43–56, 0.1 mg/kg twice daily day 57–119, 0.1 mg/kg qd day 120–180.

PREPARATIVE REGIMENS

Two preparative regimens were employed for the allo-BMT patients. Oral busulfan (Bu) 4 mg/kg/day for 4 days followed by IV cyclophosphamide (Cy) 60 mg/kg by a 1-hr intravenous infusion for 2 days was used in most patients with myelogenous leukemia [44]. The remaining patients were conditioned with intravenous Cy (50 mg/kg/day for 4 days and for 2 days in some of the matched unrelated donor transplants) followed by 300 CGy total body irradiation (TBI) given daily for 4 days [45].

TTP/HUS Patients (See Table II)

Twenty-seven adult patients with TTP/HUS were divided into two groups based upon their peak plasma creatinine level. Group I (Cr $\leq$ 2.0 mg/dl) consisted of 14 patients (4 males and 10 females) with a median age of 36 years (range = 21–89 years). The demographics for Group II (Cr > 2.0 mg/dl) were similar. This group had four males and nine females with a median age of 56 years (range = 26–86). The median platelet count at diagnosis and the degree of elevation of LD (expressed as times the upper range of normal) were similar in both

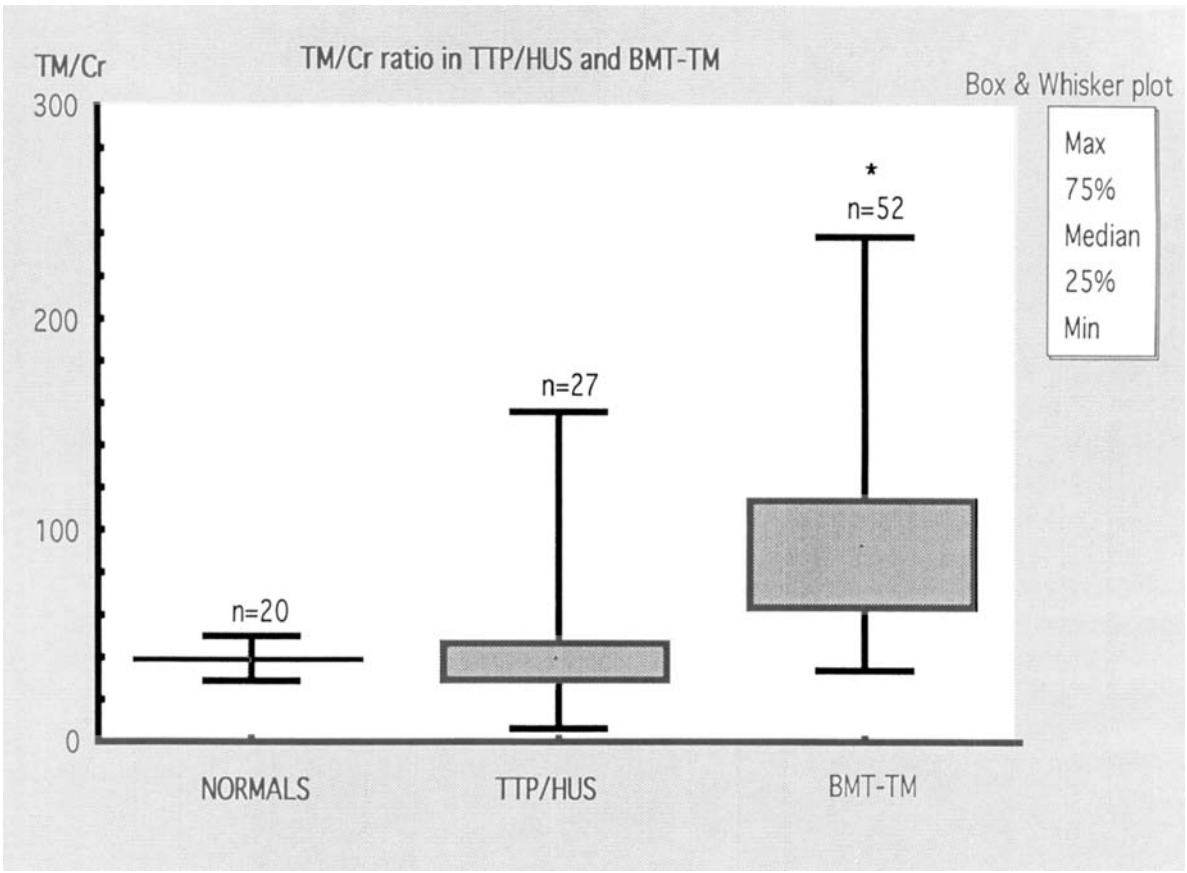

Fig. 2. Box and Whisker plot of Thrombomodulin/Creatinine (TM/Cr) ratios in TTP/HUS, BMT-TM, and Normal control subjects. n = number. *$P < .001$ vs. Normals and TTP/HUS.

groups. Eight/fourteen patients in Group I had neurologic symptoms. This consisted of transient aphasis in three, confusion and/or obtundation in four, and paresthesias in the remaining patient. Eleven out of thirteen patients in Group II had neurologic symptoms. Two patients had transient aphasia, one had aphasia and seizures, one had seizures, two patients had paresthesias, and the remaining five had confusion. The median creatinine at diagnosis in Group I was 1.3 mg/dl (range = 0.9–1.7) and in Group II was 4.0 mg/dl (range = 1.2–9.3 mg/dl), $P < 0.001$.

The TTP/HUS disorder was idiopathic in 13/14 Group I patients. One patient in Group I had inactive systemic lupus erythematosus. In Group II, the disorder was idiopathic in nine, associated with progressive systemic sclerosis (one patient), polymyositis (one patient), polymyalgia rheumatica (one patient), and mitomycin (one patient).

BMT-TM Patients

Fifty-two patients developed clinical BMT-TM at a median of 39 (range, 5–458) days post-transplant. Of these, 21 had Grade 2, 14 Grade 3, and 17 Grade 4 BMT-TM. Thirty-three of the allo-transplants were identical sibling transplants, five were non-identical, and fourteen were matched unrelated donor transplants.

The BMT-TM patients had a higher percentage of males (65%) than did the TTP/HUS patients (30%), $P = 0.003$. Fewer patients with Grade 2 BMT-TM (5%) had neurologic symptoms than did patients with TTP/HUS (70%) or patients with Grades 3 and 4 BMT-TM (74%), $P < 0.001$.

Platelet counts were consistently < 20 K/μl in the patients with Grade 4 BMT-TM and were significantly lower than those in patients with Grade 2 or 3 BMT-TM, $P < 0.001$. LD levels were increased 5.7-fold (range 2.9–14.8) in patients with Grade 4 BMT-TM. These levels were similar to those in the TTP/HUS patients and higher than those seen in patients with Grade 2 or 3 BMT-TM, $P < 0.001$. Patients with Grade 2 BMT-TM had LD levels that were increased 1.9-fold (range 1.1–6.4) and these values were lower than those seen in the non-transplant TTP/HUS patients, $P < 0.01$.

Creatinine levels at diagnosis were higher in patients with Grade 4 BMT-TM with a median Cr = 2.1 (range 1.2–6.4) compared to patients with Grade 2 BMT-TM (median = 1.2, range = 1.0–2.3), $P < 0.001$. However,

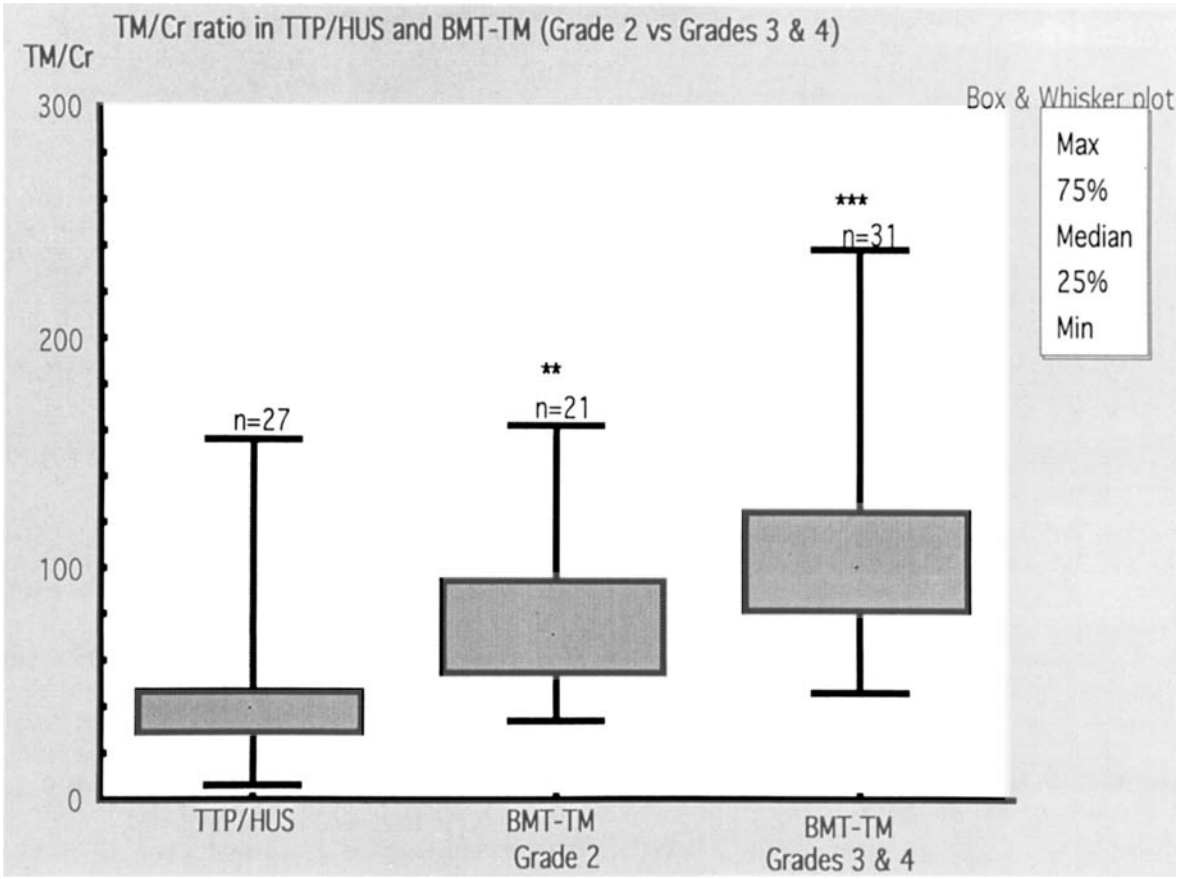

Fig. 3. Box and Whisker plot of Thrombomodulin/Creatinine (TM/Cr) ratios in TTP/HUS, Grade 2 BMT-TM, and Grades 3 and 4 BMT-TM. n = number. **$P < .001$ Grade 2 BMT-TM vs. TTP/HUS, ***$P < .001$ vs. TTP/HUS and $P = 0.004$ vs. Grade 2 BMT-TM.

TABLE I. Grading System for BMT-TM

Grade	LD (U/L)	% Fragmented cells	Clinical BMT-TM
0	Normal or increased	≤1.2	None
1	Normal	≥1.3	Subclinical
2	Increased	1.3–4.8	Mild
3	Increased	4.9–9.6	Moderate
4	Increased	≥9.7	Severe

the creatinine levels in Grade 4 BMT-TM were lower than those in patients with TTP/HUS who presented with an "HUS" picture (median = 4.0, range = 1.2–9.3), $P < 0.004$.

RESULTS

vWF:Ag in TTP/HUS, BMT-TM, and Controls

von Willebrand Factor (vWF:Ag) levels (Fig. 1) were elevated at diagnosis in patients with TTP/HUS and with BMT-TM compared to controls [(normal range = 1.21 ± 0.60 (2SD U/ml)], $P < 0.001$. vWF:Ag levels were significantly higher ($P < 0.001$) in the patients with BMT-TM (median = 4.38 U/ml with a range of 1.07–14.14 U/ml) compared to patients with TTP/HUS (median = 2.57, range = 1.21 to 10.85 U/ml). However vWF:Ag levels did not correlate with the grade of BMT-TM (data not shown). vWF:Ag levels correlated with TM/Cr ratios in TTP/HUS ($P < 0.001$) but did not correlate with TM/Cr ratios in BMT-TM ($P < 0.213$).

Thrombomodulin Levels in TTP/HUS and BMT-TM Patients

All patient groups had TM levels higher than controls [mean = 25 ng/ml with a range (mean ± 2 SD) of 9 to 50 ng/ml], $P < 0.003$. TM values were significantly ($P < 0.001$) higher in the TTP/HUS patients with peak CR > 2.0 mg/dl) (median = 213 ng/ml with a range of 97–365 ng/ml) and in the BMT-TM patients (median = 120 ng/ml, range = 45 to 735 ng/ml) than in the TTP/HUS patients with peak creatinine levels ≤ 2.0 ng/ml. The median TM values were 39 ng/ml with a range of 8 to 136 ng/ml in this latter group.

Patients with Grade 2 BMT-TM had a median TM level of 98.0 ng/ml with a range of 46–195 ng/ml. These

TABLE II. Clinical Characteristics of TTP/HUS and BMT-TM Patients at Diagnosis

Syndrome	M:F	Age	Platelets K/μL (150–400)[a]	LD X upper normal	Creatinine (MG/DL)	Neurologic symptoms
TTP/HUS (peak Cr ≤ 2.0 mg/dl)	4:10	36 (21–89)	18 (5–68)	4.1 (1.3–15.4)	1.3 (0.9–1.7)	8/14 (57%)
TTP/HUS (peak Cr > 2.0 mg/dl)	4:9	56 (25–86)	34 (4–90)	5.5 (2.3–13.1)	4.0 (1.2–9.3)	11/13 (85%)
BMT-TM (Grade 2, n = 21)	17:4	38 (19–53)	43 (20–149)	1.9 (1.1–6.4)	1.2 (1.0–2.3)	1/21 (5%)
BMT-TM (Grade 3, n = 14)	7:7	43 (17–58)	22 (20–128)	2.4 (1.2–7.4)	1.3 (0.6–2.7)	10/14 (71%)
BMT-TM (Grade 4, n = 17)	10:7	29 (20–47)	<20	5.7 (2.9–14.8)	2.1 (1.2–6.4)	13/17 (76%)

[a]Patients receiving platelet transfusions were considered to have platelets <20 K/μl. Data shown as median (range).

levels did not differ from those in patients with TTP/HUS (median TM level = 73 ng/ml with a range of 8 to 365 ng/ml). By contrast, patients with Grade 3 and 4 BMT-TM had median TM levels of 159 mg/ml with a range of 45 to 735 ng/ml. These values were significantly higher than those in TTP/HUS ($P = 0.04$) and Grade 2 BMT-TM ($P = 0.001$).

Thrombomodulin Creatinine (TM/Cr) Ratio in TTP/HUS and BMT-TM Patients

Since TM is excreted in the urine, a ratio of TM/Cr was calculated and compared for the patient groups and controls (Fig. 2). The median TM/Cr ratios did not differ between the TTP/HUS groups and controls. The median value in nine control subjects was 37.5 (range = 28.8–50.0). Similar values were observed in the TTP/HUS patients with peak Cr ≤ 2.0 mg/dl (median = 37.5 with a range of 6.2–123.6) and with peak Cr > 2.0 mg/dl (median ratio = 41.1 with a range of 14.4–156.3). Seven patients with TTP/HUS had elevated TM/Cr ratios. Of these, three were classified as TTP and four as HUS. By contrast, the TM/Cr ratio was elevated in the BMT-TM patients and differed significantly from the control patients and the TTP/HUS patients, $P < 0.001$. The median TM/Cr ratio was 91.4 ng/ml with a range of 33.8–239.2 ng/ml.

TM/Cr ratios (Fig. 3) were significantly higher (median = 70, range 34 to 163) in patients with Grade 2 BMT-TM compared to the patient with TTP/HUS (median = 38, range = 6 to 156), $P < 0.001$). In addition, patients with Grades 3 and 4 BMT-TM had higher TM/Cr ratios (median = 100, range = 46 to 239) than did patients with Grade 2 BMT-TM ($P = 0.004$) or TTP/HUS ($P < 0.001$).

DISCUSSION

The present study compared vWF:Ag and TM levels in adult patients with TTP/HUS (with and without an "HUS" presentation) to patients with BMT-TM. Both markers were elevated (compared to controls) in all patient groups. vWF:Ag and TM were higher in BMT-TM, compared to TTP/HUS. However, TM/Cr ratios did not differ from normal in either group of TTP/HUS patients. By contrast, BMT-TM patients had higher TM/Cr ratios than controls or adult patients with TTP/HUS. Moreover TM and TM/Cr correlated with the severity of BMT-TM.

Previous studies have reported elevated vWF:Ag and TM levels (at diagnosis) in adult patients with TTP/HUS [33,34]. Takahashi et al. showed a correlation between TM level and creatinine, which is in agreement with the findings of the present study [34]. They did not report TM/Cr ratios. vWF:Ag levels have been reported to correlate with grade of BMT-TM [13]; however, this was not the case in the present study. By contrast, TM and TM/Cr did correlate with BMT-TM grade. Patients with Grade 2 BMT-TM had lower TM and TM/Cr values than those patients with BMT-TM Grades 3 and 4. However, no differences were observed between Grades 3 and 4.

TM is the endothelial cell receptor for thrombin (that is pivotal in the activation of the protein C anticoagulant system) [46]. In contrast to vWF:Ag, TM is apparently released only by damage to endothelial cells [37–39]. However, TM is excreted in the urine and elevated TM levels are seen in renal failure [40]. The TM/Cr ratio has been used to account for the renal contribution to TM levels. For example, patients with lupus glomerulonephritis have higher TM/Cr ratios than patients with other nephropathies [40].

Taken together, these observations suggest that endothelial cell stimulation occurs in TTP/HUS and BMT-TM. In TTP/HUS, vWF:Ag levels correlated with TM/Cr ratios, whereas there was no correlation between vWF:Ag and TM/Cr ratio in BMT-TM. These findings indicate a dissociation between these two endothelial derived proteins.

It is unclear whether endothelial cell damage occurs in TTP/HUS or whether the high TM levels are simply

a reflection of altered renal clearance in TTP/HUS pts with an "HUS" presentation. Since TM/Cr ratios were high in BMT-TM, these results strongly suggest that endothelial cell damage (alone or in combination with altered renal clearance) contributes to the elevated TM levels in these pts. Loss of TM from endothelium may be important in the pathogenesis of BMT-TM. Whether these changes are pathogeneic in causing the BMT associated TTP/HUS disorder or reflect the development of a TTP/HUS disorder in a host who has developed endothelial damage as a result of chemoradiotherapy and/or CsA is unclear.

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